

Characterization of Lassa Virus Cell Entry and Neutralization with Lassa Virus Pseudoparticles[▽]

François-Loïc Cosset,^{1*†} Philippe Marianneau,^{2†} Geraldine Verney,¹ Fabrice Gallais,² Noel Tordo,² Eve-Isabelle Pécheur,³ Jan ter Meulen,⁴ Vincent Deubel,^{2,5} and Birke Bartosch¹

Université de Lyon, UCB-Lyon 1, IFR128, INSERM, U758, and Ecole Normale Supérieure de Lyon, Lyon F-69007, France¹; Unité de Biologie des Infections Virales Emergentes, Institut Pasteur, IFR 128 BioSciences Gerland, Lyon Sud, Lyon, France²; Institut de Biologie et Chimie des Protéines, CNRS, UMR 5086, Université Lyon 1, IFR 128 BioSciences Gerland, Lyon Sud, Lyon, France³; Institute of Virology, Philipps University, Marburg, Germany⁴; and Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China⁵

Received 12 August 2008/Accepted 28 December 2008

The cell entry and humoral immune response of the human pathogen Lassa virus (LV), a biosafety level 4 (BSL4) Old World arenavirus, are not well characterized. LV pseudoparticles (LVpp) are a surrogate model system that has been used to decipher factors and routes involved in LV cell entry under BSL2 conditions. Here, we describe LVpp, which are highly infectious, with titers approaching those obtained with pseudoparticles displaying G protein of vesicular stomatitis virus and their use for the characterization of LV cell entry and neutralization. Upon cell attachment, LVpp utilize endocytic vesicles for cell entry as described for many pH-dependent viruses. However, the fusion of the LV glycoproteins is activated at unusually low pH values, with optimal fusion occurring between pH 4.5 and 3, a pH range at which fusion characteristics of viral glycoproteins have so far remained largely unexplored. Consistent with a shifted pH optimum for fusion activation, we found wild-type LV and LVpp to display a remarkable resistance to exposure to low pH. Finally, LVpp allow the fast and quantifiable detection of neutralizing antibodies in human and animal sera and will thus facilitate the study of the humoral immune response in LV infections.

Lassa virus (LV) is a negative-strand RNA virus and belongs to the family *Arenaviridae*, which comprises many important human pathogens such as Junin virus, Machupo virus, and the prototype arenavirus lymphocytic choriomeningitis virus (LCMV). LV virions are pleiomorphic enveloped particles ranging from 50 to 300 nm in diameter and encapsidate two segments of single-stranded RNA, both containing two genes encoded in an ambisense orientation. The glycoprotein (GP) precursor (GPC) is encoded by the smaller RNA, is posttranslationally cleaved into two subunits (GP1, which is responsible for cell attachment, and GP2, harboring transmembrane and fusogenicity determinants), and is essential for α -dystroglycan receptor recognition, cell entry, and the induction of neutralizing antibodies (7, 8, 22, 30, 41).

The natural hosts of LV are rodents, and the transmission of the virus from its reservoir to humans causes approximately 150,000 cases of Lassa fever annually in West Africa, which vary in severity from mild influenza-like illness to lethal hemorrhagic fever (39). For Lassa fever, mortality is associated with a high viral load, and humoral immune responses are thought not to contribute to disease resolution, because neutralizing antibodies are generally detected in LV-infected patients only late in convalescence, and recombinant Lassa fever vaccines provide protection from lethal challenge in the absence of neutralizing antibodies (19, 26). During acute infec-

tion, rapid viral dissemination critically depends on the efficient attachment of the viruses to receptor molecules on target cells and subsequent entry and replication (31). Investigations of cell entry of LV have so far been a difficult area of research due to the high pathogenicity and biosafety level 4 (BSL4) classification of the virus. Furthermore, quantification of LV neutralizing antibodies has been challenging because of their low titers and technical limitations of the plaque reduction neutralization assay (59). To overcome these restrictions, model systems for LV cell entry have been described. These include replication-competent vesicular stomatitis virus (VSV) vectors expressing an LV GP chimera (18), recombinant LCMV expressing LV GPs (49), as well as infectious LV pseudoparticles (LVpp) consisting of retroviral core particles that display unmodified and fully functional LV GPs and mimic the overall functions of the wild-type (wt) virus (28, 47, 50).

We describe here the biochemical characterization of LVpp and report infectious titers of LVpp similar to those of pseudoparticles from the G protein of VSV (VSV-G), one of the most efficient viral GPs available for pseudotyping (3). Furthermore, we have used LVpp to characterize LV cell entry and to develop a neutralization assay for reproducible quantification of antibody targeted against LV GP function(s). Like wt LV, LVpp display a ubiquitous cell tropism, and the use of lysosomotropic agents confirmed a pH-dependent endocytic cell entry (20). We confirm that LV GPs fuse at unusually low pH values, with an optimum of fusion between pH 3 and 4 in cell-cell fusion assays (27), and demonstrate moreover that LVpp and wt LV are remarkably resistant to exposure to low pH. Finally, the infectivity of LVpp can be neutralized quantitatively with antibodies or sera targeted against the LV GPs.

* Corresponding author. Mailing address: Human Virology Department, INSERM U758, Ecole Normale Supérieure de Lyon, 46 allée d'Italie, 69364 Lyon Cedex 07, France. Phone: 33 472 72 87 32. Fax: 33 472 72 81 37. E-mail: fcosset@ens-lyon.fr.

† F.-L.C. and P.M. contributed equally to the work.

[▽] Published ahead of print on 19 January 2009.

The LVpp assay may therefore become a useful tool for the detection of neutralizing antibodies and to analyze the role of the humoral immune response in LV infection.

MATERIALS AND METHODS

Cells and viruses. Cell lines used in this study were SKHep1 cells (HTB-52), MeWo cells (HTB-65), 293T human embryo kidney cells (CRL-1573), Huh-7 human hepatocellular carcinoma cells (43), TE671 human rhabdomyosarcoma cells (CRL-8805), U118 human glioblastoma cells (HTB-15), Jurkat human T-cell leukemia cells (TIB-152), and Vero African green monkey kidney cells (CCL-81). Peripheral blood mononuclear cells (PBMCs) were purified as previously described (37). LV strain AV was cultured in Vero cells at 37°C under BSL4 conditions. Cell-free supernatant with a viral titer of 2×10^7 focus-forming units (FFU)/ml was then used as the infectious virus stock. This supernatant was also gamma irradiated (60 Co and 50 kGy) and used as a source of inactivated LV.

Expression constructs. An expression vector for the LV GPC was constructed by standard PCR using primers 5'-CGCGGATCCACCATGGGACAAATAGTG and 5'-TCGAAGGCCTATCTCTTCATTTCAC using vector pBAC Access displaying the LV GPC sequence corresponding to strain AV (GenBank accession number M15076) as a template. The resulting fragment was digested with BamHI and StuI and cloned into pcDNA3.1(-)Hygro (Invitrogen), which had been digested with HindIII and treated with Klenow fragment to fill in the HindIII site, followed by a BamHI digestion. The LV coding sequence was excised from the resulting plasmid by AflII and BamHI digestions, blunted with Klenow fragment, and cloned into the blunted BamHI sites of expression plasmid pHCMV-G. The murine leukemia virus (MLV) packaging and green fluorescent protein (GFP) transfer vectors pHCMV-RD114, pHCMVcE1E2, pHCMV-G, pHCMV-HA/NA, and pHCMV-LCMV (strain WE-HPI), expression plasmids encoding GPs of feline endogenous virus RD114, hepatitis C virus, vesicular stomatitis virus, influenza virus, and LCMB, respectively, were described previously (4, 52).

Pseudoparticle production, infection, and neutralization. 293T cells were transfected with expression vectors encoding viral GPs, retroviral core proteins, and GFP transfer vector using the CalPhos mammalian transfection kit (Clontech, France). Twenty-four hours after transfection, the medium was replaced with Dulbecco's modified Eagle's medium-10% fetal calf serum. Supernatants were harvested 24 h after medium change, filtered (0.45- μ m pore size), and used to infect Huh-7, Vero, and other target cells. Dilutions of viral supernatants were added to the cells and incubated for 3 h. After 3 h of incubation, supernatants were removed, and the infected cells were kept for 72 h before analysis of the percentage of GFP-positive cells by fluorescence-activated cell sorter (FACS) analysis. Infections were controlled by using nonenveloped particles, which resulted in background titers between 10^2 and 10^3 infectious units (IU)/ml. To test sera and antibodies for neutralization, pseudoparticle supernatants were preincubated with immunoglobulins or sera at the indicated dilutions for 45 min at room temperature and then used for infection.

Cell-cell fusion assay. To examine low-pH-induced syncytium formation, transfected 293T cells were rinsed once with phosphate-buffered saline (PBS) and then incubated with citrate buffers adjusted to the indicated pH values for 2 min. The cells were then washed with PBS and incubated with fresh culture medium for another 20 min after fusion induction. Finally, the cells were fixed with 0.5% glutaraldehyde and stained with May-Grünwald and Giemsa solutions (Sigma) to visualize the nuclei of cells according to the manufacturer's recommendations. The fusion index was defined as $(N - S)/T$, where N is the number of nuclei in syncytia, S is the number of syncytia, and T is the total number of nuclei counted.

Reagents and antibodies. Western blot analysis of cell lysates and pseudoparticles purified on 20% sucrose cushions was performed as previously described (4).

Transmission electron microscopy. Supernatants containing pseudoparticles (viral titers were 5×10^7 for LVpp, 3×10^4 for LCMV pseudoparticles [LCM-Vpp], and 1.3×10^7 for RD114 pseudoparticles [RD114pp]) were purified on sucrose cushions and concentrated 100-fold in PBS. wt LV was analyzed after gamma irradiation using undiluted supernatant. Viral preparations were spotted onto 300-mesh copper formvar- and carbon-coated electron microscopy grids (Electron Microscopy Sciences), stained with 1% uranyl acetate, and viewed with a Philips CM120 apparatus operated at 80 kV.

RESULTS

Biochemical and electron microscopic characterization of LVpp. The use of LVpp in cell entry and neutralization assays offers a number of significant advantages over the wt or recombinant forms of the wt virus not only in terms of safety but also in terms of technical flexibility. Indeed, the abortive infectious properties of LVpp ensure a one-round infection process and thus allow a linear correlation of viral particle input and infection events, which is of paramount importance, especially in neutralization assays (3). To ensure the full functionality of the LV GPs in the context of a heterologous retroviral core (9) and to validate LVpp for use in cell entry and neutralization, we have undertaken an in-depth characterization of their biochemical, morphological, and functional properties. The use of LVpp in cell infection was previously described in the literature (28, 47, 50), but no in-depth biochemical characterization of their assembly has been reported so far (47).

LVpp were produced by transfecting human 293T cells with three expression vectors encoding LV GPC, the MLV core proteins, and a packaging-competent MLV-derived genome harboring the GFP marker gene. Control pseudoparticles were generated with assembly-defective MLV core proteins (MLV-G2A) (4). Analysis of immunoblots of transfected and lysed producer cells showed that the structural components of the pseudoparticles were readily detected at the expected molecular masses: i.e., 76 kDa for GPC, 44 kDa for GP1, and 36 kDa for GP2 (Fig. 1A). MLV core proteins were detected as Gag precursors of 65 kDa that were partially processed into mature core components by the MLV protease. Detection of GPC as well as GP1 and GP2 in lysates of producer cells indicated that the cellular protease SKI-1/S1P is indeed active in 293T cells and that the maturation of GPC into GP1 and GP2 occurred with high efficiency (33, 34). Viral particles were harvested from the supernatant of transfected cells and purified by ultracentrifugation through high-density sucrose cushions. Only the cleaved forms GP1 and GP2, but no GPC, were detected in the pellets of purified virions, suggesting that GPC is not incorporated or is incorporated only at very low levels into LVpp, an observation consistent with the assembly process of wt LV (34). Comparison of the molecular masses of GP1 and GP2 present either in producer cells or in virions demonstrated that the mobility determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of GP1 present in virions was slower than that of GP1 present in producer cells, suggesting that only highly glycosylated forms of GP1 are incorporated into virions or that virion-associated GP1 is glycosylated during viral egress (17). In contrast to GP1, the molecular masses of GP2 present in producer cells or virions did not differ. Finally, the incorporation of GP1 and GP2 occurred only in the presence of wt MLV core proteins but not upon the coexpression of the assembly-deficient MLV-G2A capsid mutant (Fig. 1A), suggesting that GP1 and GP2 cannot assemble and support budding to form "empty" GP particles. Likewise, could the mature LV envelope GP complex efficiently assemble on retroviral core proteins derived from human immunodeficiency virus (HIV) type 1 (data not shown)?

Arenaviruses, including LV, were previously reported to display a large variation in size. Particle diameters have been shown to range from 40 to 200 nm for LCMV. However, the

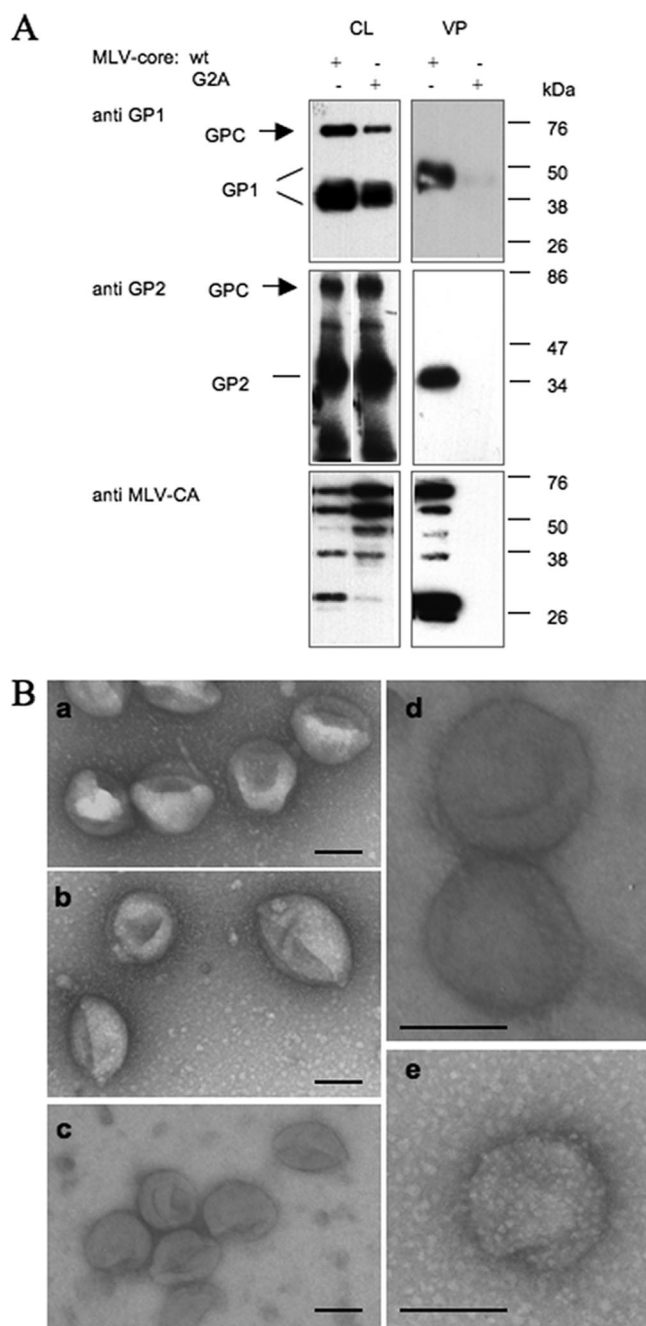


FIG. 1. Biochemical and electron microscopic analysis of LVpp. (A, left) Cell lysates (CL) of 293T cells transfected with LV GPC, MLV GFP vector, and either wt or an assembly-deficient MLV Gag/Pol mutant (G2A) were analyzed by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) and immunoblotted with antibodies against GP1 or GP2 (rabbit anti-GP2 was a kind gift from O. Lenz) or an anti-MLV core antibody (ViroMed Biosafety Laboratories). (Right) Supernatants containing viral particles (VP) were harvested from the transfected 293T cells 48 h after transfection, and the virions were purified on high-density sucrose cushions and analyzed by immunoblotting as described above. (B) Viral particles were deposited onto glow-discharged Formvar- and carbon-coated copper grids and subsequently negatively stained with 1% uranyl acetate. The electron microscope was operated under 80 kV. Images of pseudoparticles displaying the GPs of endogenous feline retrovirus RD114 (a), LCMV (b), and LV (c and d) are shown. (e) Transmission electron micrograph of wt LV displayed for comparison. Bars, 100 nm.

most prevalent particle diameter is between 80 and 90 nm (44). In contrast, retroviral particles are not uniform in diameter but nevertheless fall within a narrow distribution of sizes. Retroviral particle size is determined mainly by C-terminal Gag components and ranges between 100 and 120 nm (1). The presence of different envelope GPs influences the viral diameter only marginally (53). To investigate the morphology and homogeneity of LVpp, we negatively stained wt LV and retroviral pseudoparticles displaying the GPs of LV, LCMV, and RD114 and investigated their morphology by transmission electron microscopy. As expected, RD114pp (Fig. 1Ba), LCMVpp (Fig. 1Bb), and LVpp (Fig. 1Bc and d) displayed similar particle sizes and morphologies within a range of 90 to 130 nm (data not shown), confirming that retroviral particle size is not dramatically altered by the presence of heterologous GPs. In our hands, the diameter of wt LV (Fig. 1Be) produced in vitro in Vero cells was very comparable and also ranged between 90 and 130 nm (data not shown), which is consistent with the production of homogeneous LV particles in vitro (23, 44).

Infectivity and tropism of LVpp. LVpp produced with MLV- or HIV-derived core proteins were titrated onto Vero or Huh-7 target cells. Since the LVpp were generated with replication-defective viral components encoding a GFP marker, infectivity of the pseudoparticles could be precisely determined by FACS analysis. After incubation for 4 h, the viral supernatants were replaced with normal medium, and the infected cells were analyzed for GFP expression 72 h later. LVpp based on both MLV as well as HIV core proteins infected Vero and Huh-7 target cells with infectious titers as high as 3×10^7 iU/ml (Fig. 2A). Furthermore, LVpp could be concentrated on sucrose cushions with a only minimal loss of infectivity to reach titers of 2×10^9 iU/ml on Huh-7 cells (data not shown). Finally, no infection could be obtained with viral particles lacking GP1 and GP2 GPs, lacking core proteins (Fig. 2A), or, alternatively, when using the MLV-G2A assembly-defective core proteins (data not shown). As the infectious titers of HIV-based LVpp were generally found to be approximately four- to fivefold lower than those of MLV-based LVpp on both Huh-7 and Vero cells (Fig. 2A), MLV-based LVpp were used for subsequent experiments.

The infectious properties mediated by the LV GPs in the context of pseudoparticles were previously described to be similar to those of the wt virus; however, this has so far been verified only in terms of tropism and receptor usage (28, 47, 50). We performed receptor competition experiments between LVpp and gamma-irradiated wt LV. A supernatant containing 2×10^7 iU/ml LVpp was incubated for 1 h prior to infection with either no, 10^7 , or 10^8 PFU of irradiated wt LV or, as a control, wt yellow fever virus and subsequently used at a multiplicity of infection (MOI) of 0.2 to infect Huh-7 cells. An inhibition of LVpp infectivity was observed upon incubation with 10^8 PFU irradiated wt LV but not upon incubation with an equivalent amount of irradiated yellow fever virus, suggesting a direct competition between LVpp and wt LV in cell entry (Fig. 2B). No effect of irradiated LV particles on the entry of RD114 control pseudoparticles was observed (Fig. 2B), demonstrating that the competition was specific.

To investigate the tropism of LVpp, we used a panel of target cell lines for infection assays (Fig. 2C). Infections were controlled with pseudoparticles harboring VSV-G pseudopar-

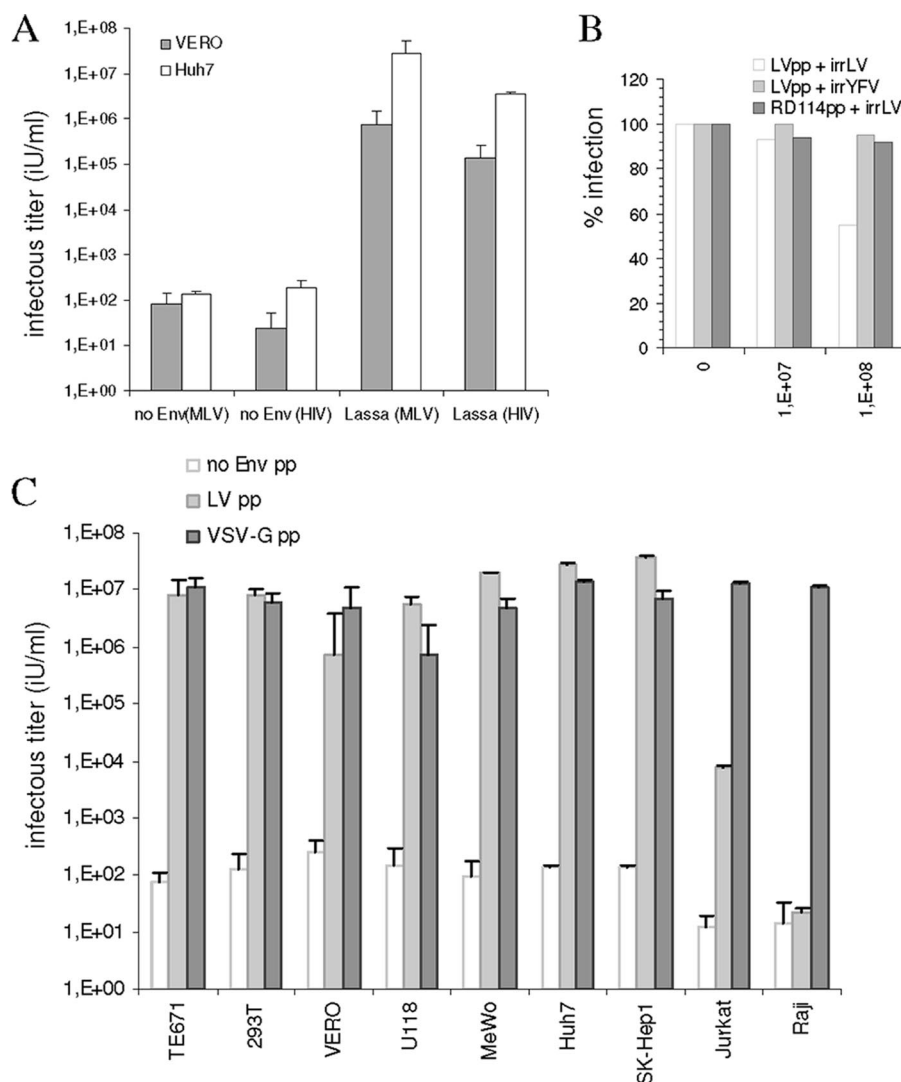


FIG. 2. Infectivity of LVpp. (A) LVpp or nonenveloped pseudoparticles were produced with viral vectors derived from either MLV or HIV (no Env [MLV], no Env [HIV], LV [MLV], and LV [HIV], respectively). Resulting pseudoparticles were titrated on Vero or Huh-7 cells. GFP expression in target cells was detected by FACS analysis 72 h after infection. Titers are displayed as iU per ml of supernatant. Standard deviations are included as error bars ($n = 5$). (B) One hundred microliters of an LVpp (2×10^7 iU/ml)- or RD114pp (6×10^6 iU/ml)-containing supernatant was preincubated with no, 10^7 , or 10^8 genome equivalents of irradiated wtLV (irrLV) or irradiated yellow fever virus (irrYFV) before infection of Vero cells. Data are standardized to pseudoparticle infection obtained in the absence of irradiated virus. Standard deviations are not included due to the difficulties in transferring irradiated viral preparations out of BSL4 containment. (C) LVpp, VSV-Gpp, and no-Env pseudoparticles were titrated on the indicated cell lines, and analysis of infection was performed as described above ($n = 3$).

ticles (VSV-Gpp) that display a very high titer and wide cell tropism. Cells were incubated for 4 h with dilutions of supernatants containing LVpp or VSV-Gpp, washed, and cultured until the expression of the GFP marker gene was measured by FACS analysis 72 h later. Infectious titers of up to 2×10^7 iU/ml were detected for LVpp on Huh-7 target cells (Fig. 2C). Infectious titers on other target cell lines were in the range of 7.3×10^5 iU/ml (Vero) to 1×10^7 iU/ml (TE671). Cell lines of hepatic (Huh-7 and SK-Hep1), endothelial (MeWo), or neuronal (U118, human glioma) origin as well as a rhabdomyosarcoma cell line (TE671) were all highly susceptible to LVpp. On the T-cell-derived lineage Jurkat, which has so far been reported to be nonpermissive to LV infection, we detected a low but reproducible titer with LVpp, as previously observed for

LCMVpp (24). No infection was observed with LVpp on Raji cells that originated from a B-cell Burkitt's lymphoma (Fig. 2C). We also used human PBMCs as target cells. In infection assays with HIV-based LVpp and VSV-Gpp as controls, no infection was observed with LVpp or VSV-Gpp on unstimulated PBMCs. Upon the addition of anti-CD3 and anti-CD28 antibodies to induce T-cell activation (37), the culture became susceptible to VSV-Gpp. At an MOI of 12, up to 26% of PBMCs were infected by VSV-Gpp. In contrast, even at MOIs as high as 20, no significant infection could be observed with LVpp (data not shown), suggesting that if LVpp infects PBMCs, this occurs at titers that are below the limit of detection applicable to our assays. In conclusion, as reported for wt LV, the tissue tropism of LVpp is very broad.

pH dependence of LVpp cell entry. The entry of enveloped viruses into target cells is mediated by fusion between the viral and cellular membranes. This process is catalyzed by a fusogenic apparatus contained within the viral surface GPs, which can be activated in a pH-dependent or -independent fashion (27). Following receptor binding, pH-dependent viruses are routed into endosomal vesicles, where fusion between viral and cellular membranes is activated by acidification. Lysosomotropic agents, which inhibit the drop of pH in endocytic particles, were previously shown to inhibit the cell entry of several arenaviruses, including LCMV and LV, suggesting that the fusion process is triggered by low pH (6, 13, 20, 21, 48, 49). We confirmed that treatment of Vero cells with bafilomycin A1 prior to and during infection inhibited infection with wt LV (data not shown). Consistently, bafilomycin A1 also inhibited, in a dose-dependent fashion, cell entry of LVpp and LCMVpp in Vero cells (Fig. 3A) as well as cell entry of control pseudoparticles displaying the pH-sensitive GPs of fowl plague virus (HApp) and VSV-Gpp as well as hepatitis C virus (4). In contrast, the infectivity of control pseudoparticles generated with the pH-insensitive GPs of cat endogenous retrovirus RD114 and amphotropic MLV (MLV-A) was not significantly affected by the drug, consistent with the pH-independent cell entry route adopted by these retroviruses (38). Similar results were obtained for Huh-7 cells and with the lysosomotropic agent chloroquine (data not shown). Thus, entries of both LVpp and wt LV into the cell are sensitive to lysosomotropic agents.

To investigate the role of acidification in the fusion process of LV in more detail, we performed cell-cell fusion assays. LV was previously described to fuse at abnormally low pHs in cell-cell fusion assays (28), but the lack of appropriate controls has so far not allowed a demonstration of the specificity of induction of viral fusion by low pH. We chose to perform the cell-cell fusion assay with 293T cells because they express the respective cellular receptors for all viral GPs that we included in the assay. To control the specificity of fusion activation, we included viral GPs that require acid pH to activate fusion, such as influenza virus hemagglutinin (HA), and, more importantly, also viral GPs that are independent of exposure to low pH and rely primarily on receptor-GP interactions for the initial triggering of fusion, such as gamma-retroviral RD114 and MLV-A Env GPs (11, 32). To investigate the role of acidification in the fusion process of LV, densely seeded 293T cells overexpressing LV or control viral GPs at their surface (data not shown) were washed and incubated with citrate buffers adjusted to pH values from pH 6 to 3 for 2 min. After the pH shock, cells were washed, recultured in Dulbecco's modified Eagle's medium, and, after a further incubation of 20 min, fixed and processed to assess syncytium formation (Fig. 3B). Syncytium formation was quantified by determining the size and number of syncytia as previously described (2). At neutral pH or pH 6, no syncytium formation was observed in any of the cell cultures, with the only exception being cells expressing the highly fusogenic RD114 GP, a GP that induces syncytium formation in a pH-independent but rather receptor-dependent manner (11, 32). Syncytium formation in cell cultures expressing pH-dependent viral GPs was observed only upon exposure to low pH. HA-mediated cell-cell fusion was strongly induced by pH 5 or 4 and reduced at pH 3. In 293T cell cultures expressing the GPCs of

LV or LCMV, maximal induction of syncytium formation required exposure to rather low pH values. Cell-cell fusion was activated upon exposure to pH 4 and even more pronounced upon exposure to pH 3, a pH value at which HA-mediated cell-cell fusion is already suboptimal (Fig. 3B) (28). To control the specificity of LCMV and LV fusion activation at low pH values, we analyzed cells expressing the MLV-A GP, which is insensitive to low pH and does not mediate syncytium formation. MLV-A-expressing cells did not show any tendency to fuse after a pH 3 shock, demonstrating for the first time that syncytium formation at this pH value was specifically induced by the viral GPs and not due to aberrant effects induced by the low-pH treatment.

Exposure to low pH in the absence of cellular receptors or lipids abolishes the infectivity of a number of viral GPs, including influenza virus HA (54, 55). The shifted pH optimum of cell-cell fusion activity of LV GPs prompted us to investigate the pH resistance of LVpp. For this purpose, we adjusted supernatants containing pseudoparticles displaying different pH-sensitive or -insensitive GPs to pH values ranging from pH 3 to pH 7 using citrate buffers. After a 5-min exposure at the indicated pH values, the pH of the supernatants was neutralized, and they were subsequently tested in infection assays (Fig. 3C). The pH-insensitive MLV-A pseudoparticles were affected only by exposure to the lowest pH value (pH 3), while RD114pp retained full infectivity even after exposure to pH 3. In contrast, the infectious titers of HApp were significantly decreased by exposure to pH values of 5 or below, as was previously reported in the literature (29). Remarkably, both LVpp as well as LCMVpp did not or only marginally display a loss of infectivity, even after exposure to pH 4. Exposure to pH 3, in contrast, very significantly reduced the respective infectivity. Importantly, we could confirm the pH resistance of wt AV and LV Josiah strains upon exposure to the same range of citrate buffers as those used for pseudoparticle pH sensitivity testing; no inhibition of cell entry and subsequent replication was detected in wt LV-infected Vero target cells by reverse transcription-PCR upon exposure to pH values of between pH 7.0 and 3.5 (Fig. 3D). Evaluation of infectivity upon exposure to pH 3.0 was not possible due to strong cellular toxicity.

Consistent with these functional infection data, transmission electron microscopy revealed no alterations of the membrane morphology for LVpp after incubation at pH 7.4 or pH 5 (Fig. 4A). Upon incubation at pH 4, differences in the membrane topology resulting in an altered shape of LVpp were detectable, while exposure to pH 3 caused membrane disintegration and rupture with a concomitant leakage of capsid material. In contrast, nonenveloped particles (data not shown) and RD114pp (Fig. 4B) displayed no morphological changes after exposure to any of the pH values tested. This finding is fully consistent with the strong pH resistance of RD114pp and fusion activation of LVpp between pH 4 and 3.

In conclusion, cell entry of LVpp and LCMVpp is clearly pH dependent, like that of HApp. However, the arenavirus GPs are significantly more pH resistant and display cell-cell fusion activity at much lower pH values than HA.

Neutralization of LV. Having demonstrated high infectivity, conserved cell entry properties, and conserved functionalities of GP1 and GP2 displayed on LVpp, we explored LVpp as a potential tool to study the humoral immune response to LV in-

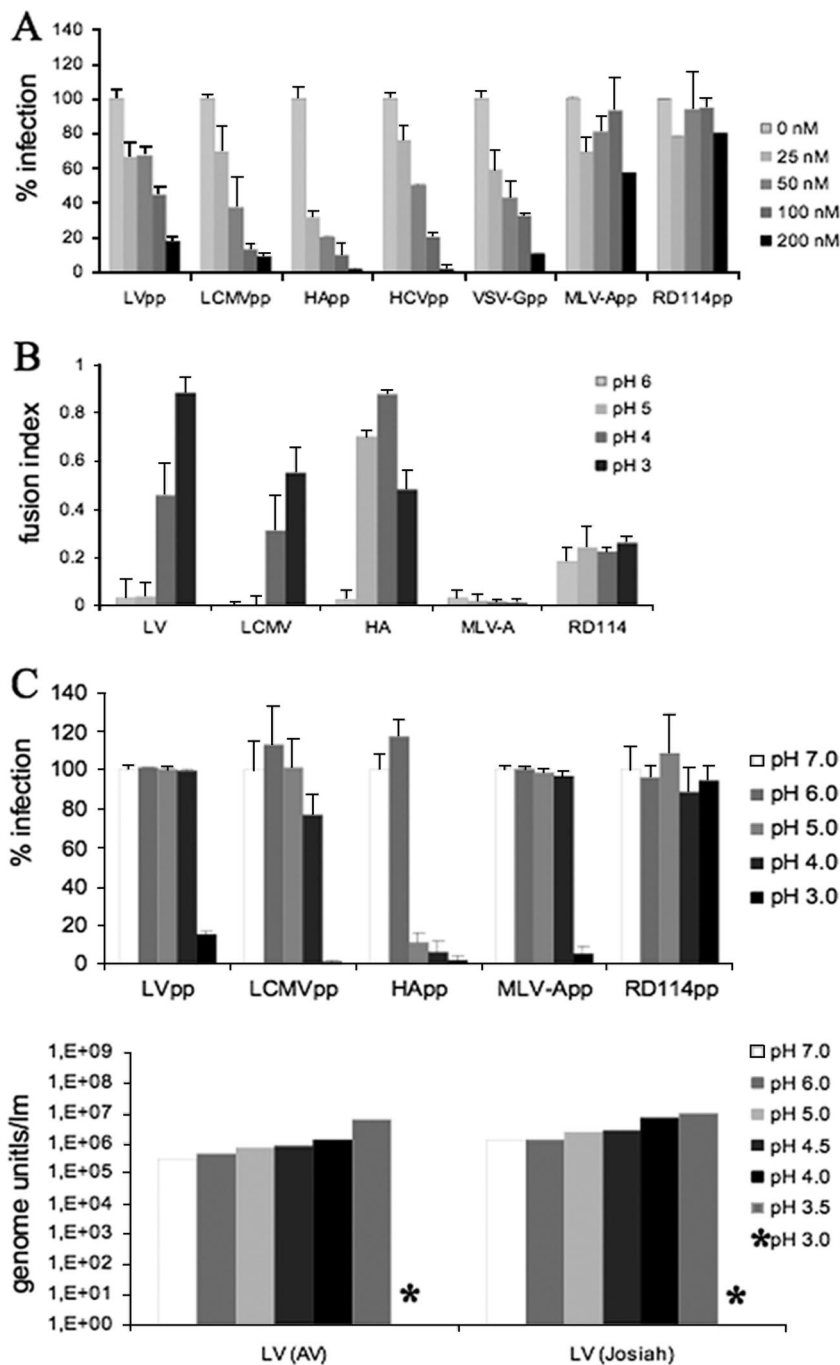


FIG. 3. pH dependence of LV cell entry and fusion. (A) Huh-7 or Vero cells were incubated with the indicated concentrations of bafilomycin A1 before (2 h) and during (3 h) infection with pseudoparticles displaying the indicated GPs (hepatitis C virus pseudoparticles [HCVpp], HApp, and MLV-A pseudoparticles [MLV-App] display the GPs of hepatitis C virus, influenza virus, and MLV-A, respectively). GFP expression in target cells was detected by FACS analysis 72 h after infection. The results are expressed as percent average infection obtained in the absence of bafilomycin A1. (B) 293T producer cells were washed and exposed to citrate buffers adjusted to the indicated pH values for 2 min. The cells were then rapidly washed, recultured for 20 min, and processed with May-Grünwald and Giemsa solutions (Sigma) according to the manufacturer's instructions in order to visualize the nuclei. The fusion index was defined as follows: $(N - S)/T$, where N is the number of nuclei in syncytia, S is the number of syncytia, and T is the total number of nuclei counted. (C) The pH of viral supernatants containing the indicated pseudoparticles (top) or wt LV strain AV versus Josiah (bottom) was adjusted using citrate buffers at the indicated pH values. After 5 min, the pH values were neutralized and used in infection assays with Huh-7 cells. Data are standardized to infection obtained with the supernatant adjusted to pH 7.

fection. We compared LVpp neutralization assays and wt LV plaque reduction tests (59) using a number of different anti-LV antibodies and sera. To exclude cell type-specific effects, most neutralizations were performed using Vero as well as Huh-7 target cells. Hybridoma supernatants of anti-LV GP1 and GP2 monoclonal antibodies (kindly provided by T. Ksiazek, CDC, Atlanta, GA) raised in mice against baculovirus-expressed and purified GPC of strain LV-1, which cross-react

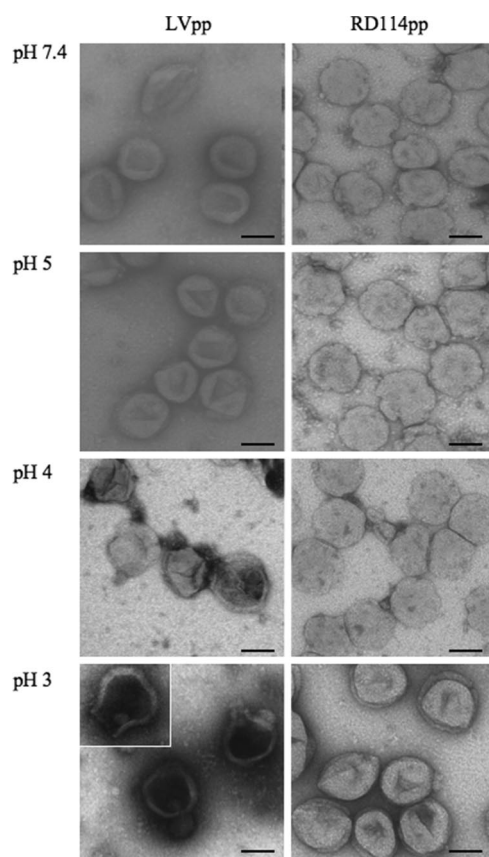


FIG. 4. Morphology of LV and RD114 pseudotyped at various pHs by transmission electron microscopy. LVpp (A) and RD114pp (B) in suspension in PBS (pH 7.4) were either deposited onto carbon/formvar-coated copper electron microscopy grids (extreme left) or preincubated at pH 5.0 (left middle), pH 4.0 (right middle), or pH 3.0 (extreme right) for 5 min before deposition onto grids and negatively stained with 1% uranyl acetate. For both panels, the top gallery gives an overview, while the bottom gallery shows representative details. Bars, 100 nm.

with the GPC of LV strain AV in enzyme-linked immunosorbent assays (ELISAs) (data not shown), were preincubated at 1/20 dilutions, with LVpp displaying AV strain-derived GPs. No inhibition of infection was observed with Vero (Fig. 5A) or Huh-7 (data not shown) cells. We also tested sera derived from eight macaques, which had developed various severities of disease following challenge with different doses of LV strain AV (P. Marianneau and V. Deubel, unpublished data). Sera were sampled 1 month postinoculation and displayed high titers against various LV antigens in immunoglobulin G ELISAs but no neutralizing activity at 1/20 dilutions with LVpp or wt LV displaying GPC of strain AV (data not shown). In contrast, a polyclonal serum derived from a goat hyperimmunized with LV strain Josiah (a kind gift from A. Chepurinov, Russia) showed a strong and dose-dependent cross-neutralizing activity (Fig. 5B). This neutralization was specific for LV, as the infectivity of control RD114pp was not affected. LVpp and wt LV were neutralized with very similar efficiencies on Vero cells, and the neutralization of LVpp was even more efficient on Huh-7 cells (Fig. 5B).

To investigate the usefulness of LVpp for the detection and

quantification of neutralizing antibodies in humans, we used ELISAs to identify suitable samples from a serum collection derived from healthy persons living in highly endemic zones in Sierra Leone or Guinea (57). Antigens used in the ELISAs were based on LV strain AV and constituted either lysates of LV-infected cells (Table 1) or recombinant, purified GPC protein (data not shown). Cutoff values for the ELISAs were set to an optical density (OD) of 0.1; we selected 10 sera with ODs above the cutoff value and also three further sera with an OD value close to or at the background of detection ($OD < 0.05$) (Table 1). Of the 13 sera, 3 displayed neutralizing activity in infection as well as plaque reduction assays, with LVpp and wt LV displaying the GPs of strain AV (Fig. 5C). While all three neutralizing sera (HS1, HS2, and HS3) had tested positive in the ELISAs, levels of total immunoglobulin G reactive with LV cell lysate did not correlate with neutralizing activity (Table 1). Interestingly, weak cross-neutralization of LCMVpp was observed with two of the sera, while cross-neutralization was more pronounced for the third serum sample (Fig. 5C). Neutralization was specific for LV and LCMV GPs, as in control reactions, the infectivity of RD114pp was not affected (Fig. 5C). Neutralization of wt LV was performed using Vero cells, while LVpp and LCMVpp were neutralized with similar efficiencies using Vero as well as Huh-7 cells.

DISCUSSION

Arenaviruses have a short replication cycle coupled to a high mutation frequency, leading to the generation of quasispecies that differ from the original isolate (51). The abortive infectivity of retroviral pseudotypes has the distinct advantage that one can directly compare the functionality of a defined viral GP sequence and analyze its infectious properties in a highly quantitative fashion in cell entry and neutralization assays. We report here infectious titers of over 2×10^7 GFP iU/ml for nonconcentrated LVpp, which makes LVpp one of the most infectious pseudotypes described to date (3). LVpp mimic the functional properties of parental LV, and their infectious properties are strictly dependent on an assembly process that allows the presence of the correctly processed LV GPs on the particle surface (33, 34). Despite the previously reported interaction of LV GPs with the underlying arenavirus Z protein (9), in a pseudoparticle context based on retroviral capsid proteins, LV GP1 and GP2 are still fully functional. Indeed, LVpp display a conserved tropism and can be directly outcompeted by irradiated wt LV. The high infectious titer of LVpp, which we describe here, may explain why we can report LVpp infection of Jurkat cells for the first time, albeit at low titers. This cell type was previously described to be refractory to LVpp infection due to the absence of detectable α -dystroglycan processing (47, 50). It remains to be investigated whether LVpp entry on Jurkat cells is due to an independence of LVpp cell entry on correctly processed α -dystroglycan or, alternatively, whether the extent of α -dystroglycan processing may vary in different Jurkat cell batches. In conclusion, the combination of its high titer, which can reach over 10^9 iU/ml after concentration, combined with its ubiquitous tissue tropism make LVpp a promising delivery tool for gene therapy approaches.

The interaction of the LV GPs with their cellular receptor α -dystroglycan and the dependence of LV cell entry on lyso-

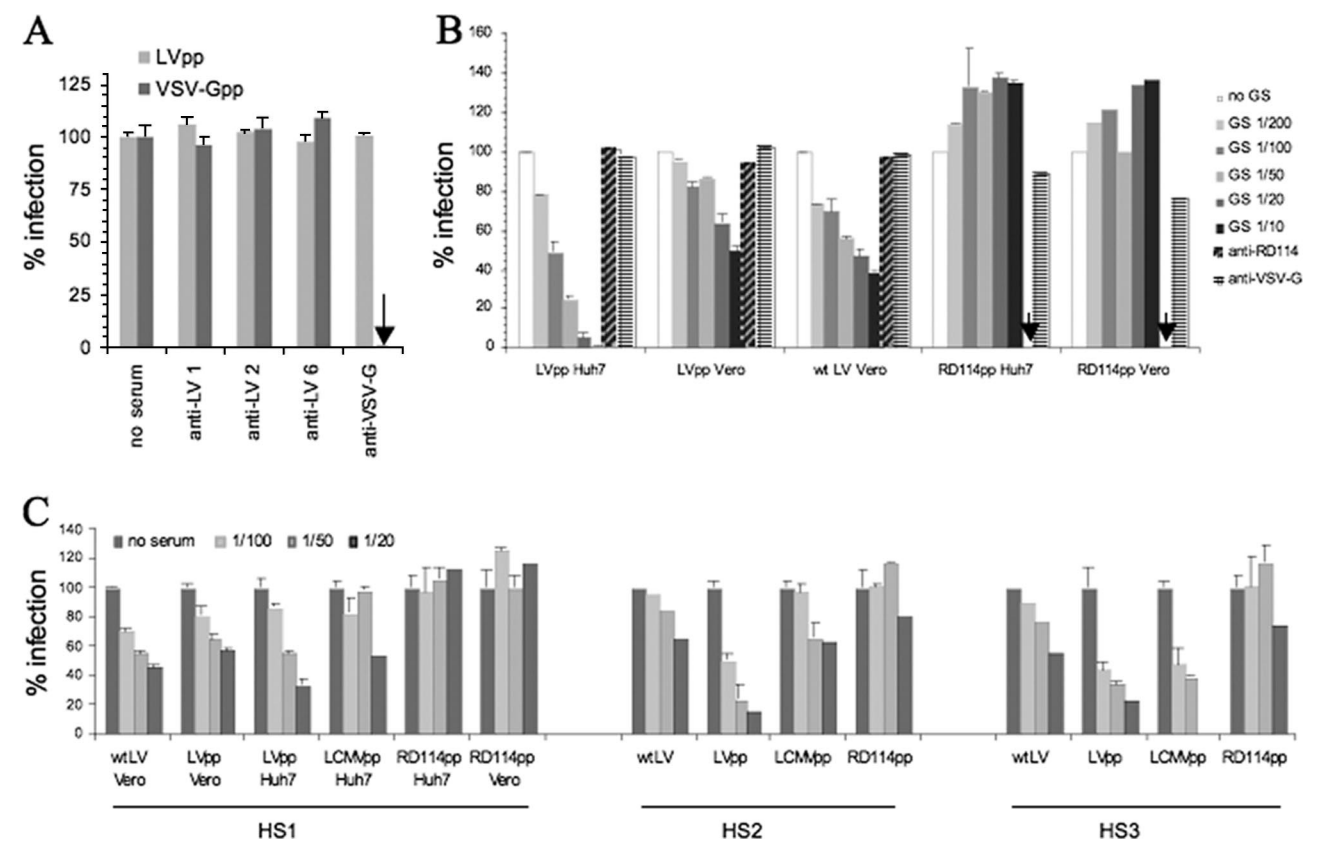


FIG. 5. Neutralization of LVpp. Infectivities of wt LV, LVpp, LCMVpp, and control RD114pp were tested in the presence of various antibodies or sera. Results are expressed as the percent average infectious titers obtained in the absence of antibodies or sera. Neutralizations were performed with hybridoma supernatants of anti-LV GP mouse monoclonal antibodies at 1/20 dilutions (A), with serum from a goat hyperimmunized with LV at the indicated dilutions (B), or with three human sera at the indicated dilutions on the indicated target cells (C). Neutralizations with HS1 were performed on Huh-7 as well as Vero cells as indicated; neutralizations with HS2 and HS3 were performed on Vero cells. Arrows indicate titers below the detection threshold.

somotropic agents are well described in the literature (6, 10, 21). However, in comparison to its close relative LCMV and other New World arenaviruses, characterization of the route of LV cell entry has only recently been described and seems to depend on an as-yet-uncharacterized endocytic pathway (49, 61), and the only report on the pH dependence of fusion

activation of the LV GP complex has only recently become public in the form of a cell-cell fusion assay (28). Unexpectedly, and in contrast to the cell-cell fusion activity of influenza virus HA and Junin virus GP, which displayed their fusion optima at pH >5, LV and LCMV GPs were reported to have maximal cell-cell fusion activity at pH <4.5 (28). We substantiate and extend these findings by demonstrating that fusion activation below pH 5 is specific, because the pH-insensitive MLV-A and RD114 GPs can withstand such treatment without changes to their respective fusogenic behaviors and particle morphologies. Furthermore, we demonstrate that LVpp, LCMVpp, as well as wt LV display resistance to pH values as low as pH 4. This pH resistance of LV and LCMV is consistent with optimal fusion activation of LV and LCMV GPCs at low pH values in cell-cell fusion and suggests that these viruses fuse in a late endocytic or possibly lysosomal compartment(s) where the pH value has been described to drop to pH 4.5 (40) and may be low enough to activate the viral fusion machinery. While the actual pH range that can occur within the cellular endocytic trafficking system remains in flux, as it is technically difficult to measure and has been explored mainly with pH-sensitive fluorescent probes (40), alternative explanations for our findings may be that multiligand interactions (60) or additional interactions of the LV GP complex with

TABLE 1. LV ELISA titers and neutralizing activity in human sera from zones where LV is endemic

Serum	LV cell lysate anti-IgG OD	Neutralization	
		LVpp	wt LV
HS3	0.55	+	+
P1	0.48	—	—
SL598	0.38	—	—
SL421	0.31	—	—
SL589	0.27	—	—
HS2	0.26	+	+
B33 10-1	0.23	—	—
HS1	0.18	+	+
B35 6-1	0.11	—	—
TB28 5-1	0.10	—	—
B13 6-3	0.03	—	—
TB11 6-1	0.03	—	—
SL424	0.02	—	—

cellular factors or lipids within endosomes or lysosomes (16, 42) modulate the viral fusion machinery and help to trigger the fusion event at more elevated pH values. Identification of the endocytic compartment in which LV fusion occurs will help to shed light on the mechanistic roles of pH in LV cell entry.

The pH resistance of LCMVpp described here is contradictory to previous findings for wt LCMV, which was previously described to undergo irreversible conformational changes upon exposure to values below pH 6, leading to viral inactivation, probably via the shedding of GP-1 (12, 13). In those studies, LCMV strain ARM was used, in contrast to strain WE, on which our study is based (52). However, it is unlikely that sequence differences, which were reported to result in altered receptor affinity and cell surface attachment, are sufficient to explain this discrepancy for several reasons (5, 24, 36, 56). We observed the same pattern of pH-induced fusion behavior and pH resistance with LCMVpp as well as LVpp and wt LV. Furthermore, while some of the pH studies of LCMV ARM implied that prolonged exposure to low pH inactivates the virus, several pieces of evidence suggest that arenavirus GPs can withstand exposure to low pH. Curiously, LCMV strain ARM has been successfully used to establish productive infections in mice upon gastric transmission (46), suggesting a certain intrinsic pH resistance. While the gastric transmission of arenaviruses and of vectors expressing arenaviral antigens (14, 15, 46) has been shown to attenuate infection, it can still lead to protective immunity from lethal disease (35). While direct contact with contaminated secretion or blood has so far been thought to be the major route of infection in West Africa, where hunting of peridomestic rodents and consumption of their meat was shown to be a risk factor for LV infection (58), our findings raise the question of whether the pH resistance of LV may form an adaptation to possible oral transmission through the gastrointestinal tract.

Cellular immunity plays a primary role in recovery from LV infection (31), while the significance of the humoral immune response in viral clearance remains unclear. A limited number of studies of humans (26, 59) as well as guinea pigs and non-human primates (25, 45) suggest that neutralizing antibody may not contribute to convalescence due to their late appearance. However, a protective effect of neutralizing antibody against LV infection has been demonstrated in vaccination and antibody therapy studies (31). To investigate the humoral immune response to LV and the causes for the delayed appearance of neutralizing antibody in disease, seroepidemiological studies are needed. These are most easily performed within the zones of endemicity and require a performant and a robust assay system. To this end, we investigated the suitability of LVpp in a serum neutralization assay. A goat hyperimmunized with LV Josiah did produce significant amounts of antibodies that cross-neutralized wt LV strain AV and LVpp displaying AV-derived GPs. A group of macaques that were inoculated with various doses of LV AV (the associated pathology and cellular immune responses displayed by the animals will be described elsewhere) were also tested for the development of neutralizing antibody. After a 30-day incubation, during which a number of animals displayed signs of infection, animals were sacrificed, and serum samples were collected. No neutralizing antibody could be detected using LVpp displaying homologous

LV GPC sequences, suggesting that in the macaque model, neutralizing antibody development is delayed or impaired. Among the human serum samples that we identified to contain anti-LV antibodies in ELISAs using cell lysates and recombinant GP derived from LV strain AV, only three displayed detectable levels of neutralizing antibody in LVpp (AV) neutralization and wt LV (AV) plaque reduction assays (Fig. 5C and Table 1). Among the three sera that displayed neutralizing activity, two were derived from patients with previously confirmed LV infections. In both these patients, strong cellular immune responses and antibody titers had been detected for many years (57). Importantly, cross-neutralization of LCMVpp was observed with all three sera, albeit to various levels. The establishment of pseudoparticles displaying the GPs of different LV and other Old and New World arenaviruses will help to shed light on the issue of cross-neutralization and the identification of (cross)neutralizing epitopes.

While further characterization of LVpp is required, overall, these particles mimic the functional properties of parental wt LV closely and are technically easy to produce and manipulate. LVpp may therefore be suitable to further decipher LV cell entry and to explore vaccination strategies in the future.

ACKNOWLEDGMENTS

We thank T. Ksiazek, P. Jahrling, O. Lenz, and A. Chepurinov for kindly providing sera and antibodies. Experiments involving infectious Lassa virus have been realized in the BSL4 laboratory of Jean Mérieux, INSERM, Lyon, France. Electron microscopy was performed at the Centre Technologique des Microstructures at Villeurbanne-La Doua, Université Claude Bernard Lyon 1. We thank Els Verhoeven for technical help with PBMC activation and Sandra Lacôte, Christelle Granier, Judith Fresquet, and Maude Michelet for technical assistance.

This work was supported by grants from the European Union (LSHB-CT-2004-005246 COMPUVAC and LSHB-CT-2004-005242 CONSERT), the European Research Council (ERC Advanced Grant no. 233130 "HEPCENT" to F.-L.C.), the Agence Nationale de Recherches sur le SIDA et les Hépatites Virales, Aventis Pharma (Sanofi-Aventis group), and Bayer Pharma as part of a multiorganism call for proposals (Biotox/Prothemo).

We have no conflicting financial interests.

REFERENCES

1. Ako-Adjei, D., M. C. Johnson, and V. M. Vogt. 2005. The retroviral capsid domain dictates virion size, morphology, and coassembly of Gag into virus-like particles. *J. Virol.* **79**:13463–13472.
2. Andersen, K. B. 1994. A domain of murine retrovirus surface protein gp70 mediates cell fusion, as shown in a novel SC-1 cell fusion system. *J. Virol.* **68**:3175–3182.
3. Bartosch, B., and F. L. Cosset. 2004. Strategies for retargeted gene delivery using vectors derived from lentiviruses. *Curr. Gene Ther.* **4**:427–443.
4. Bartosch, B., J. Dubuisson, and F. L. Cosset. 2003. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J. Exp. Med.* **197**:633–642.
5. Beyer, W. R., H. Miletic, W. Ostertag, and D. von Laer. 2001. Recombinant expression of lymphocytic choriomeningitis virus strain WE glycoproteins: a single amino acid makes the difference. *J. Virol.* **75**:1061–1064.
6. Borrow, P., and M. B. Oldstone. 1994. Mechanism of lymphocytic choriomeningitis virus entry into cells. *Virology* **198**:1–9.
7. Buchmeier, M. J. 2002. Arenaviruses: protein structure and function. *Curr. Top. Microbiol. Immunol.* **262**:159–173.
8. Cao, W., M. D. Henry, P. Borrow, H. Yamada, J. H. Elder, E. V. Ravkov, S. T. Nichol, R. W. Compans, K. P. Campbell, and M. B. Oldstone. 1998. Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. *Science* **282**:2079–2081.
9. Capul, A. A., M. Perez, E. Burke, S. Kunz, M. J. Buchmeier, and J. C. de la Torre. 2007. Arenavirus Z-glycoprotein association requires Z myristoylation but not functional RING or late domains. *J. Virol.* **81**:9451–9460.
10. Castilla, V., S. E. Mersich, N. A. Candurra, and E. B. Damonte. 1994. The entry of Junin virus into Vero cells. *Arch. Virol.* **136**:363–374.
11. Cheynet, V., A. Ruggieri, G. Oriol, J. L. Blond, B. Boson, L. Vachot, B.

- Verrier, F. L., Cosset, and F. Mallet. 2005. Synthesis, assembly, and processing of the Env ERVWE1/syncytin human endogenous retroviral envelope. *J. Virol.* **79**:5585–5593.
12. Di Simone, C., and M. J. Buchmeier. 1995. Kinetics and pH dependence of acid-induced structural changes in the lymphocytic choriomeningitis virus glycoprotein complex. *Virology* **209**:3–9.
 13. Di Simone, C., M. A. Zandonatti, and M. J. Buchmeier. 1994. Acidic pH triggers LCMV membrane fusion activity and conformational change in the glycoprotein spike. *Virology* **198**:455–465.
 14. Djavani, M., C. Yin, I. S. Lukashevich, J. Rodas, S. K. Rai, and M. S. Salvato. 2001. Mucosal immunization with *Salmonella typhimurium* expressing Lassa virus nucleocapsid protein cross-protects mice from lethal challenge with lymphocytic choriomeningitis virus. *J. Hum. Virol.* **4**:103–108.
 15. Djavani, M., C. Yin, L. Xia, I. S. Lukashevich, C. D. Pauza, and M. S. Salvato. 2000. Murine immune responses to mucosally delivered *Salmonella* expressing Lassa fever virus nucleoprotein. *Vaccine* **18**:1543–1554.
 16. Earp, L. J., S. E. Delos, R. C. Netter, P. Bates, and J. M. White. 2003. The avian retrovirus avian sarcoma/leukosis virus subtype A reaches the lipid mixing stage of fusion at neutral pH. *J. Virol.* **77**:3058–3066.
 17. Eichler, R., O. Lenz, W. Garten, and T. Strecker. 2006. The role of single N-glycans in proteolytic processing and cell surface transport of the Lassa virus glycoprotein GP-C. *Virol. J.* **3**:41.
 18. Garbutt, M., R. Liebscher, V. Wahl-Jensen, S. Jones, P. Moller, R. Wagner, V. Volchkov, H. D. Klenk, H. Feldmann, and U. Stroher. 2004. Properties of replication-competent vesicular stomatitis virus vectors expressing glycoproteins of filoviruses and arenaviruses. *J. Virol.* **78**:5458–5465.
 19. Geisbert, T. W., S. Jones, E. A. Fritz, A. C. Shurtleff, J. B. Geisbert, R. Liebscher, A. Grolla, U. Stroher, L. Fernando, K. M. Daddario, M. C. Guttieri, B. R. Mothe, T. Larsen, L. E. Hensley, P. B. Jahrling, and H. Feldmann. 2005. Development of a new vaccine for the prevention of Lassa fever. *PLoS Med.* **2**:e183.
 20. Glushakova, S. E., A. I. Iakuba, A. D. Vasiuchkov, R. F. Mar'iankova, T. M. Kukareko, T. A. Stelmakh, T. P. Kurash, and I. S. Lukashevich. 1990. Lysosomotropic agents inhibit the penetration of arenaviruses into a culture of BHK-21 and Vero cells. *Vopr. Virusol.* **35**:146–150. (In Russian.)
 21. Glushakova, S. E., and I. S. Lukashevich. 1989. Early events in arenavirus replication are sensitive to lysosomotropic compounds. *Arch. Virol.* **104**:157–161.
 22. Gunther, S., and O. Lenz. 2004. Lassa virus. *Crit. Rev. Clin. Lab. Sci.* **41**:339–390.
 23. Howard, C. R., and P. R. Young. 1984. Variation among New and Old World arenaviruses. *Trans. R. Soc. Trop. Med. Hyg.* **78**:299–306.
 24. Imperiali, M., C. Thoma, E. Pavoni, A. Brancaccio, N. Callewaert, and A. Oxenius. 2005. O-mannosylation of α -dystroglycan is essential for lymphocytic choriomeningitis virus receptor function. *J. Virol.* **79**:14297–14308.
 25. Jahrling, P. B. 1983. Protection of Lassa virus-infected guinea pigs with Lassa-immune plasma of guinea pig, primate, and human origin. *J. Med. Virol.* **12**:93–102.
 26. Jahrling, P. B., J. D. Frame, J. B. Rhoderick, and M. H. Monson. 1985. Endemic Lassa fever in Liberia. IV. Selection of optimally effective plasma for treatment by passive immunization. *Trans. R. Soc. Trop. Med. Hyg.* **79**:380–384.
 27. Kielian, M., and F. A. Rey. 2006. Virus membrane-fusion proteins: more than one way to make a hairpin. *Nat. Rev. Microbiol.* **4**:67–76.
 28. Kewitz, C., H. D. Klenk, and J. ter Meulen. 2007. Amino acids from both N-terminal hydrophobic regions of the Lassa virus envelope glycoprotein GP-2 are critical for pH-dependent membrane fusion and infectivity. *J. Gen. Virol.* **88**:2320–2328.
 29. Krumbiegel, M., A. Herrmann, and R. Blumenthal. 1994. Kinetics of the low pH-induced conformational changes and fusogenic activity of influenza hemagglutinin. *Biophys. J.* **67**:2355–2360.
 30. Kunz, S., P. Borrow, and M. B. Oldstone. 2002. Receptor structure, binding, and cell entry of arenaviruses. *Curr. Top. Microbiol. Immunol.* **262**:111–137.
 31. Kunz, S., and J. C. de la Torre. 2005. Novel antiviral strategies to combat human arenavirus infections. *Curr. Mol. Med.* **5**:735–751.
 32. Lavillette, D., M. Marin, A. Ruggieri, F. Mallet, F. L. Cosset, and D. Kabat. 2002. The envelope glycoprotein of human endogenous retrovirus type W uses a divergent family of amino acid transporters/cell surface receptors. *J. Virol.* **76**:6442–6452.
 33. Lenz, O., J. ter Meulen, H. Feldmann, H. D. Klenk, and W. Garten. 2000. Identification of a novel consensus sequence at the cleavage site of the Lassa virus glycoprotein. *J. Virol.* **74**:11418–11421.
 34. Lenz, O., J. ter Meulen, H. D. Klenk, N. G. Seidah, and W. Garten. 2001. The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. *Proc. Natl. Acad. Sci. USA* **98**:12701–12705.
 35. Lukashevich, I. S., M. Djavani, J. D. Rodas, J. C. Zapata, A. Usborne, C. Emerson, J. Mitchen, P. B. Jahrling, and M. S. Salvato. 2002. Hemorrhagic fever occurs after intravenous, but not after intragastric, inoculation of rhesus macaques with lymphocytic choriomeningitis virus. *J. Med. Virol.* **67**:171–186.
 36. Matloubian, M., S. R. Kolhekar, T. Somasundaram, and R. Ahmed. 1993. Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. *J. Virol.* **67**:7340–7349.
 37. Maurice, M., E. Verhoeven, P. Salmon, D. Trono, S. J. Russell, and F. L. Cosset. 2002. Efficient gene transfer into human primary blood lymphocytes by surface-engineered lentiviral vectors that display a T cell-activating polypeptide. *Blood* **99**:2342–2350.
 38. McClure, M. O., M. A. Sommerfelt, M. Marsh, and R. A. Weiss. 1990. The pH independence of mammalian retrovirus infection. *J. Gen. Virol.* **71**:767–773.
 39. McCormick, J. B., and S. P. Fisher-Hoch. 2002. Lassa fever. *Curr. Top. Microbiol. Immunol.* **262**:75–109.
 40. Mellman, I., R. Fuchs, and A. Helenius. 1986. Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* **55**:663–700.
 41. Meyer, B. J., J. C. de la Torre, and P. J. Southern. 2002. Arenaviruses: genomic RNAs, transcription, and replication. *Curr. Top. Microbiol. Immunol.* **262**:139–157.
 42. Mothes, W., A. L. Boerger, S. Narayan, J. M. Cunningham, and J. A. Young. 2000. Retroviral entry mediated by receptor priming and low pH triggering of an envelope glycoprotein. *Cell* **103**:679–689.
 43. Nakabayashi, H., K. Taketa, T. Yamane, M. Oda, and J. Sato. 1985. Hormonal control of alpha-fetoprotein secretion in human hepatoma cell lines proliferating in chemically defined medium. *Cancer Res.* **45**:6379–6383.
 44. Neuman, B. W., B. D. Adair, J. W. Burns, R. A. Milligan, M. J. Buchmeier, and M. Yeager. 2005. Complementarity in the supramolecular design of arenaviruses and retroviruses revealed by electron cryomicroscopy and image analysis. *J. Virol.* **79**:3822–3830.
 45. Peters, C. J., P. B. Jahrling, C. T. Liu, R. H. Kenyon, K. T. McKee, Jr., and J. G. Barrera Oro. 1987. Experimental studies of arenaviral hemorrhagic fevers. *Curr. Top. Microbiol. Immunol.* **134**:5–68.
 46. Rai, S. K., B. K. Micales, M. S. Wu, D. S. Cheung, T. D. Pugh, G. E. Lyons, and M. S. Salvato. 1997. Timed appearance of lymphocytic choriomeningitis virus after gastric inoculation of mice. *Am. J. Pathol.* **151**:633–639.
 47. Reigner, T., J. Oldenburg, B. Noble, E. Lamb, V. Romanowski, M. J. Buchmeier, and P. M. Cannon. 2006. Receptor use by pathogenic arenaviruses. *Virology* **353**:111–120.
 48. Rojek, J. M., M. Perez, and S. Kunz. 2008. Cellular entry of lymphocytic choriomeningitis virus. *J. Virol.* **82**:1505–1517.
 49. Rojek, J. M., A. B. Sanchez, N. T. Nguyen, J. C. de la Torre, and S. Kunz. 2008. Different mechanisms of cell entry by human-pathogenic Old World and New World arenaviruses. *J. Virol.* **82**:7677–7687.
 50. Rojek, J. M., C. F. Spiropoulou, K. P. Campbell, and S. Kunz. 2007. Old World and clade C New World arenaviruses mimic the molecular mechanism of receptor recognition used by α -dystroglycan's host-derived ligands. *J. Virol.* **81**:5685–5695.
 51. Ruiz-Jarabo, C. M., C. Ly, E. Domingo, and J. C. de la Torre. 2003. Lethal mutagenesis of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV). *Virology* **308**:37–47.
 52. Sandrin, V., B. Boson, P. Salmon, W. Gay, D. Negre, R. Le Grand, D. Trono, and F. L. Cosset. 2002. Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and nonhuman primates. *Blood* **100**:823–832.
 53. Sandrin, V., P. Boulanger, F. Penin, C. Granier, F. L. Cosset, and B. Bartosch. 2005. Assembly of functional hepatitis C virus glycoproteins on infectious pseudoparticles occurs intracellularly and requires concomitant incorporation of E1 and E2 glycoproteins. *J. Gen. Virol.* **86**:3189–3199.
 54. Sato, S. B., K. Kawasaki, and S. Ohnishi. 1983. Hemolytic activity of influenza virus hemagglutinin glycoproteins activated in mildly acidic environments. *Proc. Natl. Acad. Sci. USA* **80**:3153–3157.
 55. Szeesi, J., B. Boson, P. Johnsson, P. Dupeyrot-Lacas, M. Matrosovich, H. D. Klenk, D. Klatzmann, V. Volchkov, and F. L. Cosset. 2006. Induction of neutralising antibodies by virus-like particles harbouring surface proteins from highly pathogenic H5N1 and H7N1 influenza viruses. *Virol. J.* **3**:70.
 56. Teng, M. N., P. Borrow, M. B. Oldstone, and J. C. de la Torre. 1996. A single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with the ability to cause growth hormone deficiency syndrome. *J. Virol.* **70**:8438–8443.
 57. ter Meulen, J., M. Badusche, K. Kuhnt, A. Doetze, J. Satoguina, T. Marti, C. Loeliger, K. Koulemou, L. Koivogui, H. Schmitz, B. Fleischer, and A. Hoerauf. 2000. Characterization of human CD4+ T-cell clones recognizing conserved and variable epitopes of the Lassa virus nucleoprotein. *J. Virol.* **74**:2186–2192.
 58. Ter Meulen, J., I. Lukashevich, K. Sidibe, A. Inapogui, M. Marx, A. Dorlemann, M. L. Yansane, K. Koulemou, J. Chang-Claude, and H. Schmitz. 1996. Hunting of peridomestic rodents and consumption of their meat as possible risk factors for rodent-to-human transmission of Lassa virus in the Republic of Guinea. *Am. J. Trop. Med. Hyg.* **55**:661–666.
 59. Tomori, O., K. M. Johnson, M. P. Kiley, and L. H. Elliott. 1987. Standardization of a plaque assay for Lassa virus. *J. Med. Virol.* **22**:77–89.
 60. Ukkonen, P., V. Lewis, M. Marsh, A. Helenius, and I. Mellman. 1986. Transport of macrophage Fc receptors and Fc receptor-bound ligands to lysosomes. *J. Exp. Med.* **163**:952–971.
 61. Vela, E. M., L. Zhang, T. M. Colpitts, R. A. Davey, and J. F. Aronson. 2007. Arenavirus entry occurs through a cholesterol-dependent, non-caveolar, clathrin-mediated endocytic mechanism. *Virology* **369**:1–11.